The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

#3

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed P. Mahoney

Dated 28 August 2003

Patents Form 1/77

Patents Act 1977
(Rule 16)

THE PATENT OFFICE
13 AUG 2002
RULE 97
NEWPORT

02 E741153-1 C88300
P01/7700 0.00-0218951.2

1/77

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

The Patent Office

Cardiff Road
Newport
South Wales
NP10 8QQ

1. Your reference

Anti-infective diseases

2. 0218951.2

13 AUG 2002

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Haptogen Ltd.
Polwarth Bldg.
Foresterhill
Aberdeen
AB25 2ZD
(Incorporated in Scotland, UK)

8445108001

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention

Methods for the Control, Treatment and Management of Infectious Bacterial Disease

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Dr Keith Charlton
Haptogen Ltd.
Polwarth Building
Foresterhill
Aberdeen
AB25 2ZD

KILBURN & STRODE
20 RED LION STREET
LONDON
WC1R 4PJ
125001

8445108001

Patents ADP number *(if you know it)*

FS1/7
27.02.

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))

YES

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	0
Description	25
Claim(s)	0
Abstract	1
Drawing(s)	5+S

CF

10. If you are also filing any of the following, state how many against each item.

Priority documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	#1 ✓
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

Signature Dr. K Charlton Date 9/08/02 09/08/02

12. Name and daytime telephone number of person to contact in the United Kingdom

Dr Keith Charlton (01224) 555889
07817 039609

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

a) *If you need help to fill in this form or you have any questions, please contact the Patent Office on 08459 500505.*

b) *Write your answers in capital letters using black ink or you may type them.*

c) *If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.*

d) *If you have answered 'Yes' Patents Form 7/77 will need to be filed.*

e) *Once you have filled in the form you must remember to sign and date it.*

f) *For details of the fee and ways to pay please contact the Patent Office.*

Fields of the invention

The present invention relates to methods for controlling and treating bacterial infections in patients. The methods of the invention are applicable to most, if not all gram negative and gram-positive bacterial infections. The invention provides for the application of therapies based upon, in the preferred embodiment, immunoglobulin or immunoglobulin-like receptor molecules that have affinity and specificity for signalling molecules involved in the processes of bacterial cell to cell communication. By binding to such molecules, the receptors can be used to diagnose the presence of bacteria or to assess the disease state of patients, and can further be used to control concentrations of molecules involved in inducing a virulent state in opportunistic and other pathogens.

Background of the invention

One of the major causes of mortality and morbidity amongst patients undergoing treatment in hospitals today is due to hospital acquired infection. Susceptibility to such infection can be as a result of the primary illness for which the patient was admitted, of immuno-suppressive treatment regimes, or as a consequence of injury resulting in serious skin damage, such as burns. The bacterium to which the highest proportion of cases is attributed is *Pseudomonas aeruginosa*. It is the epitome of an opportunistic pathogen of humans. The bacterium almost never infects uncompromised tissues, yet there is hardly any tissue that it cannot infect, if the tissue defences are compromised in some manner. Although accounting for a

relatively small number of species, it poses a serious threat to human health and is used hereafter as a representative example of an infectious bacterium, and does not in any way limit the scope or extent of the present invention.

Ps. aeruginosa is an opportunistic pathogen that causes urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteraemia and a variety of systemic infections, particularly in victims of severe burns, and in cancer and AIDS patients who are immunosuppressed. Respiratory infections caused by *Ps. aeruginosa* occur almost exclusively in individuals with a compromised lower respiratory tract or a compromised systemic defence mechanism. Primary pneumonia occurs in patients with chronic lung disease and congestive heart failure. Bacteraemic pneumonia commonly occurs in neutropenic cancer patients undergoing chemotherapy. Lower respiratory tract colonisation of cystic fibrosis patients by mucoid strains of *Ps. aeruginosa* is common and difficult, if not impossible, to treat. It causes bacteraemia primarily in immuno-compromised patients. Predisposing conditions include haematologic malignancies, immuno-deficiency relating to AIDS, neutropenia, diabetes mellitus, and severe burns. Most *Pseudomonas* bacteraemia is acquired in hospitals and nursing homes where it accounts for about 25 percent of all hospital acquired gram-negative bacteraemias.

The bacterium is notorious for its natural resistant to many antibiotics due to the permeability barrier afforded by its outer membrane LPS and is, therefore, a particularly

dangerous and dreaded pathogen. Also, its tendency to colonise surfaces in a biofilm form makes the cells impervious to therapeutic concentrations of antibiotics. Since its natural habitat is the soil, living in association with the bacilli, actinomycetes and moulds, it has developed resistance to a variety of their naturally occurring antibiotics. Moreover, *Pseudomonas* spp. maintain antibiotic resistance plasmids, both R-factors and RTFs, and are able to transfer these genes by means of the bacterial processes of transduction and conjugation. Only a few antibiotics are effective against *Pseudomonas*, including fluoroquinolone, gentamicin and imipenem, and even these antibiotics are not effective against all strains. Combinations of gentamicin and carbenicillin are reportedly effective in patients with acute *Ps. aeruginosa* infections. The futility of treating *Pseudomonas* infections with antibiotics is most dramatically illustrated in cystic fibrosis patients, virtually all of whom eventually become infected with a strain that is so resistant it cannot be treated. Because of antibiotic resistance, susceptibility testing of clinical isolates is mandatory.

Ps. aeruginosa can usually be isolated from soil and water, as well as the surfaces of plants and animals. It is found throughout the world, wherever these habitats occur, so it is quite a "cosmopolitan" bacterium. It is sometimes present as part of the normal flora of humans, although the prevalence of colonisation of healthy individuals outside the hospital is relatively low (estimates range from 0 to 24 percent depending on the anatomical locale). In hospitals it is known to colonise food, sinks, taps, mops,

respiratory equipment surgical instruments. Although colonisation usually precedes infections by *Ps. aeruginosa*, the exact source and mode of transmission of the pathogen are often unclear because of its ubiquitous presence in the environment. Amongst intensive care patients in whom infection is suspected on clinical grounds, as many as 50% have no identifiable source for infection. Currently 1,400 deaths worldwide are caused each day by *Ps. aeruginosa* in intensive care units (ICU's), making it the No 1 killer.

Ps. aeruginosa is primarily a nosocomial pathogen. According to the CDC, the overall incidence of *Ps. aeruginosa* infections in US hospitals averages about 0.4 percent (4 per 1000 discharges), and the bacterium is the fourth most commonly isolated nosocomial pathogen accounting for 10.1% of all hospital-acquired infections. Globally it is responsible for 16% of nosocomial pneumonia cases, 12% of acquired urinary tract infections, 8% of surgical wound infections and 10% of bloodstream infections. Immuno-compromised patients such as neutropenic cancer and bone marrow transplant patients are susceptible to opportunistic *Ps. aeruginosa* infection, leading to 30% reported deaths. It is also responsible for 38% of ventilator-associated pneumonias and 50% of deaths amongst AIDS patients. In burns cases *Ps. aeruginosa* infections have declined in recent years due to improved treatment and dietary changes. Mortality rates however remain high, accounting for 60% all deaths due to secondary infection of burns patients.

One reason for the versatility of *Ps. aeruginosa* is that it produces a diverse battery of virulence determinants

including elastase, LasA protease, alkaline protease, rhamnolipids, type IV pilus-mediated twitching motility, pyoverdin (Williams *et al.*, 1996, Stintzi *et al.*, 1998, Glessner *et al.*, 1999), pyocyanin (Brint & Ohman, 1995, Reimmann *et al.*, 1997) and the cytotoxic lectins PA-I and PA-II (Winzer *et al.*, 2000). It is now known that many of these virulence determinants are regulated at the genetic level in a cell density-dependent manner through quorum sensing. *Ps. aeruginosa* possesses at least two quorum sensing systems, namely the *las* and *rhl* (*vsm*) systems which comprise of the LuxRI homologues LasRI (Gambello & Iglewski, 1991) and RhlRI (VsmRI) (Latifi *et al.*, 1995) respectively. LasI directs the synthesis of 3-oxo-C12-HSL (Passador *et al.*, 1993, Pearson *et al.*, 1994) whereas RhlI directs the synthesis of C4-HSL) (Winson *et al.*, 1995). The *las* and the *rhl* systems are thought to exist in a hierarchy where the *las* system exerts transcriptional control over RhlR (Williams *et al.*, 1996, Pesci *et al.*, 1997). The transcriptional activator LasR functions in conjunction with 3-oxo-C12-HSL to regulate the expression of the genes encoding for the virulence determinants elastase, LasA protease, alkaline protease and exotoxin A (Gambello & Iglewski, 1991, Toder *et al.*, 1991, Gambello *et al.*, 1993, Pearson *et al.*, 1994) as well as *lasI*. Elastase is able to cleave collagen, IgG and IgA antibodies, complement, and facilitates bacterial adhesion onto lung mucosa. In combination with alkaline protease it also causes inactivation of gamma Interferon (INF) and Tumour Necrosis Factor (TNF). LasI directs the synthesis of 3-oxo-C12-HSL which together with LasR, binds to the *lasI* promoter and creates a positive feedback system. The RhlR transcriptional activator, along with its cognate AHL (C4-

HSL), regulates the expression of *rhlAB* (rhamnolipid), *lasB*, *aprA*, RpoS, cyanide, pyocyanin and the lectins PA-I and PA-II (Ochsner *et al.*, 1994, Brint & Ohman, 1995, Latifi *et al.*, 1995, Pearson *et al.*, 1995, Winson *et al.*, 1995, Latifi *et al.*, 1996, Winzer *et al.*, 2000). These exist in a hierarchical manner where by the LasR/3-oxo-C12-HSL regulates *rhlR* (Latifi *et al.*, 1996, Pesci *et al.*, 1997) and consequently both systems are required for the regulation of all the above virulence determinants.

A number of different approaches are being actively pursued to develop therapeutics for the treatment or prevention of *Ps. aeruginosa* infection. Some are intended to be broad ranging while others are directed at specific types of *Pseudomonas* infection. Those that follow traditional routes include the development of vaccines such as that described in US patent 6,309,651, and a new antibiotic drug (SLIT) that is hoped will be effective against gram-negative bacteria in general but is designed primarily to act against *Ps. aeruginosa* and is administered by aerosol inhalation. A further observation under investigation is that the antibiotic erythromycin administered at sub-optimal growth inhibitory concentrations simultaneously suppresses the production of *Ps. aeruginosa* haemagglutinins, haemolysin, proteases and homoserine lactones (HSLs), and may be applicable for the treatment of persistent *Ps. aeruginosa* infection. Cream formulations containing amphipathic peptides are also being examined as a possible means of preventing infection of burns or other serious skin wounds. US patent 6,309,651 also teaches that antibodies against the PcrV virulence protein of *Ps. aeruginosa* may afford protection against infection.

There is also some interest in the modulation of homoserine lactone levels as a means of controlling pathogenicity. Certain algae have been demonstrated to produce competitive inhibitors of acyl-homoserine lactones (AHL's) such as furanones (Manefield, 1999), as have some terrestrial plants. These compounds displace the AHL signal molecule from its receptor protein and can act as agonist or antagonist in AHL bioassays (Tepletski et al., 2000). Other methods employed to reduce HSL concentration include the development of auto-inducer inactivation enzymes (AiiA's) that catalyse the degradation of HSLs.

There are a number of potential problems and limitations associated with the therapies currently under development. It is as yet unproven as to whether vaccines will be efficacious treatments. *Ps. aeruginosa* produces an extensive mucoid capsule that effectively protects against opsonisation by host antibodies, as revealed by patients with persistent infections having high serum titres of anti-*Pseudomonas* antibodies. A limitation in the applicability of treatments such as vaccines and anti-PcrV antibodies, as described in US patent 6,309,651, is that these approaches restrict themselves to *Pseudomonas* infection, and would not be efficacious against other bacteria. The use of auto-inducer mimics are limited by the concentrations of most that are required to effectively compete against HSLs for the receptor binding site, and the possibility of side effects. It is well known that HSLs released by Pseudomonads and other bacteria have a number of direct effects on human physiology. These include inhibition of histamine release as described in patent WO

01/26650. Patent WO 01/74801 teaches that HSLs are also able to inhibit lymphocyte proliferation and down-regulate the secretion of TNF-α by monocytes and macrophages, so acting as a general immuno-suppressant. There is a danger therefore that therapies involving the use of competitive HSL mimics may result in down-regulation of the patient's immune system. This would be generally undesirable, and particularly so in immuno-compromised patients. The use of antibiotics can, at best, be viewed as a short-term strategy in view of the remarkable ability of this bacterium (and others) to develop resistance to antibiotics.

That the pathogenesis of *Ps. aeruginosa* is clearly multifactoral is underlined by the large number of virulence factors and the broad spectrum of diseases associated with this bacterium. Many of the extra-cellular virulence factors required for tissue invasion and dissemination are controlled by cell-to-cell signalling systems involving homoserine lactone-based signal molecules and specific transcriptional activator proteins. These regulatory systems allow *Ps. aeruginosa* to adapt to a virulent form in a co-ordinated cell density dependent manner, and to overcome host defence mechanisms. Interference with such cell signalling and the associated production of virulence factors is a promising therapeutic approach to reducing illness and death caused by *Ps. aeruginosa*. The importance of such approaches is highlighted by the growing number of bacterial pathogens found to utilise similar cell-to-cell signalling systems.

There is a need to develop effective means of modulating the concentrations of HSLs and other bacterial cell signalling molecules involved in pathogenicity by methods that do not have adverse side effects, and are unlikely to be evaded by pathogenic bacteria in the foreseeable future.

Summary of the invention

The present invention provides for methods for controlling the virulence of human, animal and plant pathogenic bacteria by regulating the extra-cellular concentrations of bacterial cell signalling molecules. Whereas other treatments are restricted to a particular pathogen or group of pathogens, or to specific aspects of bacterial virulence, the present invention addresses bacterial virulence in general. The methods of the invention can be applied to short or long-term, acute or chronic illness/disease, and is effective against most or all bacterial pathogens of plants, animals and humans. The invention can also be used as a prophylactic treatment for the prevention of disease onset in individuals at risk of or from exposure to pathogenic bacteria. The invention also has the potential to limit or prevent the down-regulation of the immune system that results from many infections, and is of particular concern with patients suffering from cancer, cystic fibrosis, AIDS and other immuno-suppressive conditions. Furthermore, as the methods of the invention are directed particularly at bacterial cell signalling molecules, and not primarily at the bacterial cells themselves, there will be no selective

pressure exerted on bacterial populations to develop resistance to the treatments described.

In one preferred embodiment, the present invention is a method for inhibiting bacterial infection by administering an effective dose of anti-(bacterial) cell-signalling molecule antibody to individuals or patients at risk of infection.

Preferably, the patient is completely protected from infection.

In another embodiment, the antibody is administered to infected patients in order to modulate and reduce bacterial infection. This will include inhalation of the antibody in an aerosol by cystic fibrosis patients to increase life expectancy.

In yet another embodiment the antibody is administered to immuno-suppressed patients in order to increase immuno-competence.

In yet another embodiment conjugates of cell signalling molecules to immunogenic proteins can be administered to individuals or patients in order to stimulate an immune response against the signalling molecule resulting in the generation of neutralising antibodies.

In yet another embodiment the antibody is used as an immuno-diagnostic reagent to detect the presence of, and/or pathogenic status of potential pathogens, for example in the bloodstream or pleural fluids of patients.

In yet another embodiment the antibody is used as an immuno-capture reagent to selectively remove bacterial cell signalling molecules from patient's blood in a form of dialysis.

In yet another embodiment alternative methods can be applied to the removal of bacterial cell-cell signalling molecules from the blood of a patient with a view to modulating the pathogenicity and virulence of infecting micro-organisms. This can be achieved with other natural receptors or molecules based on natural that bind to said signal molecules. Alternatively non-natural receptors can be applied such as molecularly imprinted polymers (MIPs). This class of receptor have already been shown to be able to bind specifically to small molecular weight bio-molecules such as drugs (Hart et al., 2000) and steroids (Whitcombe et al.,1995; Ramstrom et al., 1996; Rachkov et al., 2000). In a further alternative dialysis can be achieved by the non-specific removal of all small molecular weight molecules from the patient's blood as is kidney dialysis.

In yet another embodiment the antibody is used in one or more of the above applications in combination, or in combination with other therapies, for example antibiotics, to provide additive and enhanced therapeutic regimes, disease monitoring and treatment management.

Other objects, features and advantages of the present invention, including but not limited to related applications in plant and animal hosts, will be apparent to

those skilled in the art after review of the specification and claims of the invention.

Disclosure of the invention

A growing number of bacterial species are being found to communicate between cells using a variety of small signal molecules. Gram-positive bacteria use short peptides, and gram-negative bacteria predominantly use *N*-acyl homoserine lactones. The latter are a group of compounds that share a common homoserine lactone ring structure and vary in the length and structure of a side chain. There are two classes within the group (Figure 1), the acyl-homoserine lactones (AHLs) and the oxohexanoyl-homoserine lactones (OHHLs). A single species can produce and respond to members of both classes. The cells use the molecules as a means of determining the local cell density, such that in conditions of low cell density the concentration of signal molecule is correspondingly low. In high cell densities the local signal molecule concentration is high. When this concentration reaches a threshold level it induces the transcription of genes involved in virulence and the onset of a disease state in the host.

In order to generate anti-bacterial signal molecule antibodies, it is preferable to conjugate the target molecule, or a suitable derivative, to two different carrier molecules (proteins), though a single conjugated species can be also used. Bacterial signal molecules, in general, are too small to stimulate an immune response *in-vivo*, to or be used directly as a source of antigen for the selection of high affinity antibodies from antibody

libraries. Selection of antibodies specific for the cell signalling molecular (hereafter referred to as 'antigen') is carried out in the preferred embodiment using a repertoire (library) of first members of specific binding pairs (sbp), for example a library of antibodies displayed on the surface of filamentous bacteriophage. Any other system that allows for the selection of specific receptors from a library of receptors is also applicable for the methods of the present invention. In alternative embodiments signal molecule-specific clones can be selected from a panel of antibody secreting hybridoma cell lines generated from an animal immunised with an antigen conjugate. For the purposes of a general illustration the example of a library of antibody binding sites displayed on phage particles will be used.

General method

A conjugate comprising an antigen coupled to a suitable carrier molecule, which can be a protein, a peptide or any natural or synthetic compound or material (referred to hereafter as 'conjugate-1') is immobilised onto a suitable solid support such as an 'immunotube' or microtitre plate, and the uncoated surface blocked with a non-specific blocking agent such as dried milk powder. A library of first members of sbp's ('the library') is applied to the immobilised conjugate and incubated for sufficient time for sbp members recognising conjugate-1 to bind. Phage not recognising the conjugate are removed by stringent washing. Phage that remain bound are eluted, for example with tri-ethylamine or other suitable reagent, into a buffer solution to restore neutral pH. Recovered phage particles

are then infected into a suitable host organism, e.g. *E. coli* bacteria, and cultured to amplify numbers of each selected member and so generate a second 'enriched' library. The process is then repeated using the enriched library to select for phage-antibodies ('phage') recognising the antigen conjugated to a second carrier protein (conjugate-2).

Additional rounds are performed as required, the selection process being altered to favour selection of those sbp members recognising the free form of the antigen. Phage are selected against antigen conjugates as described previously, using initially conjugate-1, and alternating with conjugate-2 (where available) for each subsequent round. Bound phage are eluted by incubating with a solution of free antigen, or antigen conjugated to small soluble selectable moieties, e.g. biotin, for sufficient time for sbp members with higher affinity for the bound form of the antigen to dissociate from the immobilised conjugate. Those phage eluted with free antigen are infected into *E. coli* cells for amplification and re-selection, and those remaining bound to the immobilised antigen discarded. Alternatively, but less preferably, all antibodies binding to conjugate may be eluted eg. with low pH.

Individual (monoclonal) phage clones from each round of selection are screened for desired binding characteristics. This can be performed by a variety of methods that will be familiar to those with ordinary skill in the art, depending on requirements, including such techniques as SPR (Surface Plasmon Resonance) and ELISA (Enzyme Linked Immuno-Sorbant

Assay). Selection criteria will include the ability to bind preferentially to the free soluble form of the antigen in the presence of conjugated derivatives.

In the preferred embodiment of the invention, antibodies will be generated from a naïve human antibody phage display library (McCafferty et al., Nature 348: 552-554, 1990; and as disclosed in WO 92/01047). Thus the antibodies could be used for administering to patients in addition to use as diagnostic or dialysis reagents. In a diagnostic assay the antibody could be used to determine the presence and concentration of HSLs in patients and so predict the patient's infection status. In other embodiments a library can be constructed from an animal pre-immunised with one or more conjugates of a HSL and a suitable carrier molecule. A further alternative is the generation of hybridoma cell lines from an animal immunised as described above. In the latter two cases it is preferable that steps be taken to reduce the immunogenicity of resulting antibodies, for example by creating host animal-human chimaeric antibodies, or "humanisation" by CDR grafting onto a suitable antibody framework scaffold. Other methods applicable will include the identification of potential T-cell epitopes within the antibody, and the subsequent removal of these e.g. by site-directed mutagenesis (de-immunisation). In a further embodiment the antibody can be engineered to include constant regions from different classes of human immunoglobulin (IgG, IgA, etc.) and produced as a whole antibody molecule in animal cells. In particular these approaches are desirable where the antibodies are to be used therapeutically.

For the present invention, the antibody may be monoclonal or polyclonal. The antibodies may be human or humanised, or for dialysis / diagnostic applications may be from other species. Antibody fragments or derivatives, such as Fab, F(ab').sup.2, Fv, or scFv, may be used, as may single-chain antibodies (scAb) such as described by Huston et al. (Int. Rev. Immunol. 10: 195-217, 1993), domain antibodies (dAbs) or antibody-like single domain antigen-binding receptors. In addition to antibodies, antibody fragments and immunoglobulin-like molecules, peptidomimetics or non-peptide mimetics can be designed to mimic the binding activity of antibodies in preventing or modulating bacterial infection by inhibiting the binding of cell-signalling molecules.

The antibodies (or equivalent) could be administered to treat bacterial infection, or used as a preventative measure for those at high risk of infection. In the case where infection already exists, the antibodies may be administered alone or in combination with anti-bacterial antibodies or antibiotics or other anti-microbial treatments. Administration of anti-HSL antibodies in conjunction with other therapies may allow the use of shorter courses or lower doses of therapeutics, so decreasing the risk of resistance arising and improving patient compliance.

It will be apparent to those of ordinary skill in the art that the compositions and methods disclosed herein may have application across a wide range of organisms in inhibiting, modulating, treating or diagnosing disease or conditions resulting from infection. The compositions and methods of

effect of residual HSL in the eluates determined from the subsequent stimulation of the bacterial cultures to fluoresce as measured by RLU. ScAbs G3B12 and G3G2 are HSL-specific, anti-VZV is specific for a viral protein, anti-Paraquat and anti-Atrazine are specific for herbicides with molecular weights similar to HSL molecules, and the Resin control contained no immobilised scAb. Data represents the means of three replicate samples from two separate assays. Standard errors are indicated.

Figure 3 shows the inhibitory effects of specific and irrelevant single-chain antibodies on the dDHL-mediated stimulation of an *E. coli* surrogate of *Ps. aeruginosa* (JM109-pSB1075) as measured by bioluminescence output. Data is given for three HSL-specific scAbs; G4H3 (●), G3G2 (■) and G3B12 (□), for the irrelevant anti- V scAb (○) (specific for a pathogenic bacterial surface protein), and in the absence of scAb (▽). Data points represent the means of three replicate samples from replicate assays.

Figure 4 shows the inhibitory effects of specific and irrelevant single-chain antibodies on the tDHL-mediated stimulation of an *E. coli* surrogate of *Ps. aeruginosa* (JM109-pSB1075) as measured by bioluminescence output. Data is given for three HSL-specific scAbs; G4H3 (●), G3G2 (■) and G3B12 (□), for the irrelevant anti- V scAb (○) (specific for a pathogenic bacterial surface protein), and in the absence of scAb (▽). Data points represent the means of three replicate samples from replicate assays.

the present invention are described with reference to *Pseudomonas aeruginosa*, but it is within the competence of one of ordinary skill in the art to apply the objects herein to other species.

The invention will now be further described by reference to the non-limiting example and figures detailed below.

Description of Figures

Table 1 shows a summary of the sensitivities (IC_{50}) of anti-AHL scAbs to fee antigen (dDHL) and to two AHL analogues (tDHL and OHHL) in competition with dDHL-BSA as determined by competitive inhibition ELISA assay.

Table 2 shows a comparison of the kinetics of two anti-AHL scAbs binding to immobilised dDHL-BSA conjugate as determined by Surface Plasmon Resonance using a BIAcore 2000 instrument. The association constants (ka), dissociation constants (kd) and affinity constants (KA, KD) are given.

Figure 1 shows the chemical structures of three representative homoserine lactone bacterial cell signalling molecules.

Figure 2 shows a comparison of the abilities of HSL-specific and irrelevant single-chain antibodies immobilised onto an inert column matrix to remove HSL from solution by immuno-affinity capture. Column eluates were applied to an *E. coli* surrogate of *Vibrio fischeri* (JM107-pSB401) and the

effect of residual HSL in the eluates determined from the subsequent stimulation of the bacterial cultures to fluoresce as measured by RLU. ScAbs G3B12 and G3G2 are HSL-specific, anti-VZV is specific for a viral protein, anti-Paraquat and anti-Atrazine are specific for herbicides with molecular weights similar to HSL molecules, and the Resin control contained no immobilised scAb. Data represents the means of three replicate samples from two separate assays. Standard errors are indicated.

Figure 3 shows the inhibitory effects of specific and irrelevant single-chain antibodies on the dDHL-mediated stimulation of an *E. coli* surrogate of *Ps. aeruginosa* (JM109-pSB1075) as measured by bioluminescence output. Data is given for three HSL-specific scAbs; G4H3 (●), G3G2 (■) and G3B12 (□), for the irrelevant anti- V scAb (○) (specific for a pathogenic bacterial surface protein), and in the absence of scAb (▽). Data points represent the means of three replicate samples from replicate assays.

Figure 4 shows the inhibitory effects of specific and irrelevant single-chain antibodies on the tDHL-mediated stimulation of an *E. coli* surrogate of *Ps. aeruginosa* (JM109-pSB1075) as measured by bioluminescence output. Data is given for three HSL-specific scAbs; G4H3 (●), G3G2 (■) and G3B12 (□), for the irrelevant anti- V scAb (○) (specific for a pathogenic bacterial surface protein), and in the absence of scAb (▽). Data points represent the means of three replicate samples from replicate assays.

Example 1

The examples described herein relate to *Vibrio fisheri* and *Pseudomonas aeruginosa*. These are given only as an example, the scope of the invention not being limited to the example but including all bacterial cell-to-cell signalling molecules that directly or indirectly regulate expression of genes involved in virulence or pathogenicity, and also including other signal molecule-induced phenotypic changes to bacterial cells such as but not limited to bioluminescence.

A derivative of a HSL was synthesised (designated dDHL-COOH), having a twelve-carbon acyl chain acting as a 'linker', and terminating in a carboxylic acid group (see Figure 1). This was conjugated, via the carboxylic acid group, to the carrier proteins Bovine Serum Albumin (BSA) and Keyhole Limpet Haemocyanin (KLH) to produce dDHL-BSA and dDHL-KLH.

The term 'linker' refers to any chemical group used to allow attachment of the hapten (antigen) to a (preferably) immunogenic carrier molecule such that the hapten is displayed away from the surface of the carrier.

In alternative objects of the invention, other carrier molecules such as magnetic beads or biotin, and other linkers and conjugation strategies can be employed. The two conjugated forms of dDHL were then used to screen an antibody phage display library. Briefly, the library was screened for a total of 3 rounds of bio-panning. In each round a dDHL-conjugate was immobilised onto a solid support

and incubated with the library of phage-antibodies for sufficient time for phage-antibodies recognising the conjugate to bind. Unbound phage were removed by stringent washing with PBS (Phosphate Buffered Saline) and PBS-Tween, and the remaining bound phage eluted by incubation at low pH (round 1). Eluted phage were then infected into *E. coli* bacteria and amplified by methods familiar to those practised in the art. The resulting amplified library of enriched clones was then used for the following round of panning. In order to reduce the numbers of clones selected that recognised the carrier protein, the immobilised conjugate (dDHL-BSA or dDHL-KLH) was alternated with successive rounds of selection. In order to bias selection in favour of clones recognising a specific HSL, the chosen HSL (dDHL) was used to competitively elute phage-antibodies during rounds 2 and 3, rather than low pH. Individual phage clones from round 3 were screened by ELISA: Each clone was assayed initially for the ability to bind to each of the dDHL-conjugates and to the carrier proteins alone. Those clones able to bind both conjugates but unable to bind either carrier protein were further assayed to identify those whose binding to conjugate could be inhibited by the presence of free dDHL in solution. The antibody variable region genes from those phage clones found to bind to free dDHL were sub-cloned into a soluble expression vector (pIMS 147), and produced as soluble single-chain antibody fragments (scAb) comprising the variable heavy and light chain domains joined by a flexible peptide linker, and a kappa constant domain from a human antibody. Quantification of the binding of soluble scAb to free HSLs was determined by competitive inhibition ELISA. Samples containing a constant concentration of each

selected scAb (with respect to 1 microgram per ml dDHL-BSA) were incubated with a range of concentrations of free dDHL (or dDHL-conjugate) for 1 h, then applied to an ELISA plate coated with dDHL-BSA. After 1 h incubation, unbound scAb was washed off and any scAb remaining bound to the immobilised conjugate detected with enzyme-labelled anti-human kappa antibody. The sensitivity of scAb for free dDHL, and cross reactivity with other HSLs (tDHL and OHHL) was determined from the concentration of free antigen that reduced the binding of scAb (without free antigen) to dDHL-BSA by 50% (IC_{50}) (Table 1).

The binding kinetics for anti-HSL scabs binding to dDHL-BSA was determined using a BIAcore 2000 (BIAcore, Sweden). A CM5 chip was activated with 0.2 M EDC [1-3-(3-dimethyl-aminopropyl)carbodiimide-HCl] / 0.05 M NHS (*N*-hydroxy-succinimide), and dDHL-BSA or BSA alone coupled to the chip in 10 nM Na-acetate at pH 3.5 or 4.5 respectively. A series of 10 concentrations of scAb (100 to 1000 nM) were assayed in duplicate in HBS buffer at a flow rate of 20 microlitres/min. Between samples the chip was regenerated with 20 microlitres 100 mM NaOH. Kinetics were determined using the BIAevaluation 3 software package (Table 2).

The ability of the scAb G3B12 to bind to OHHL was further assessed by immobilising scAb to nickel-sepharose beads in a column via a 6 x histidine tag, and passing a solution of OHHL through the column. Any OHHL bound by the scAb and retained on the column was subsequently eluted. The concentration of OHHL in the column flow though (i.e. unbound) and that bound and later eluted were determined.

The ability of the scAbs to bind to HSLs and to modulate the response of bacteria to AHLs was determined using *E. coli* strains JM107 containing the plasmid pSB401 (*Vibrio fischeri* response surrogate) and JM109 containing the plasmid pSB1075 (*Pseudomona aeruginosa* response surrogate). The reporter plasmids contain the HSL response regulator genes *luxR* (*V. fischeri*) or *las*R (*Ps. aeruginosa*), and the *luxI* promoter region, which together with exogenous HSLs activates expression of the *luxCDABE* gene fusion (the luminescence structural genes) from *Photorhabdus luminescens*. Under the appropriate growth conditions these cells are induced to emit light in response to the presence of extra-cellular HSLs, the intensity of light emitted being proportional to the concentration of HSL.

Soluble scAbs from clones selected from the library were expressed using published protocols (Strachan et al., 1998). During immobilised metal affinity chromatography purification (IMAC), scAb G3B12 was not eluted from the nickel-sepharose column. A series of additional scAbs with specificities to irrelevant antigens were also expressed and immobilised onto nickel-sepharose columns to act as controls. Five hundred microlitres of 10 nM *N*-(3-oxo)-homoserine lactone (OHHL) was applied to each column and incubated for 1 hour at 4°C. Columns were centrifuged at 40 g for 15 s and the flow through collected. Any bound OHHL was eluted with 250 microlitres 1 M NaCl. The original flow through was re-applied and incubated as before, the flow through collected and bound OHHL eluted with 1 M NaCl.

Samples of OHHL solution prior to and after passage through the immobilised scAb column were applied to *E. coli* JM107 pSB401 cultures and the light emitted measured with a luminometer. Appropriate control experiments were carried out using a column to which no scAb had been immobilised, and three additional columns including scAb with specificity's for irrelevant antigens. Cells were grown shaking at 37°C for 18 h in LB medium containing tetracycline. One millilitre of the culture was inoculated into 100 ml LB tetracycline medium and grown at 37°C until an OD 600 nm 0.2 was achieved. One hundred microlitres of the culture was applied to replicate wells of a 96-well black bio-assay plate, and an equal volume of HSL solution added. HSL solutions were 10 nM OHHL (positive control), milli-Q water passed through a nickel-sepharose column (resin control), or the flow through from passing 10 nM OHHL over columns containing immobilised scAb as described above. Plates were incubated at 37°C for 2 h with shaking, and luminescence read using an Anthos LUCY1 luminometer for 1 s (Figure 2).

The ability of the G3B12 scAb to reduce bacterial responses to HSL was assessed by HSL-inducible luminescence reporter bioassay over a period of 3.0 h using *E. coli* strain JM109-pSB1075. This strain is essentially as described for JM107-pSB401, the difference being that plasmid pSB1075 includes the *las*R of *Pseudomonas aeruginosa* in place of the *lux*R of *Vibrio fischeri*. Single colonies of JM109-pSB1075 were inoculated into 10 ml LB broth with antibiotic and incubated overnight at 37°C. Two hundred microlitres of overnight culture were inoculated into 10 ml fresh medium

and incubated at 37°C with shaking to OD 600 nm 0.2. HSL was added to the cultures (dDHL at 20 nM final conc'n or tDHL at 50 nM final conc'n) and one hundred microlitres of culture was added to triplicate wells of a black 96 well plate. No HSL was added to negative controls. Either 50 microlitres PBS or 50 microlitres scAb at 2 mg/ml was added to each well and the plate incubated further for three hours, after which time luminescence was measured at 30 min intervals and the effect of scAb on cell signalling determined (Figures 3 and 4). The data demonstrates the ability of anti-HSL antibodies to cross react with a number of structurally different homoserine lactone signal molecules, and to reduce or eliminate the response of a *Ps. aeruginosa* surrogate to extra-cellular HSL.

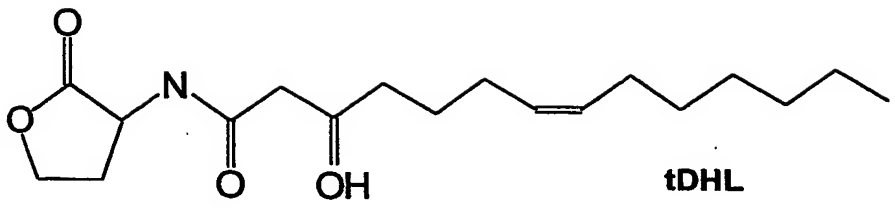
Methods for the Control, Treatment and Management of Infectious Bacterial Disease

Abstract

The present invention relates to methods for the control of virulence of infectious bacteria by modulating the extra-cellular concentration of bacterial cell signalling molecules. Derivatives of cell signalling molecules are conjugated to suitable carrier proteins and used to isolate high affinity receptors recognising the native signal molecule(s). By binding to signalling molecules, the receptors reduce and maintain extra-cellular concentrations of signal molecules below the threshold level that would otherwise result in certain opportunistic pathogens adopting a virulent form, and can transform virulent organisms to non-virulent states. These receptors have applications for the treatment of individuals with susceptibility to infection, the treatment of patients with existing infections, in disease monitoring and management, and in related applications where the host for infection is an animal or plant.

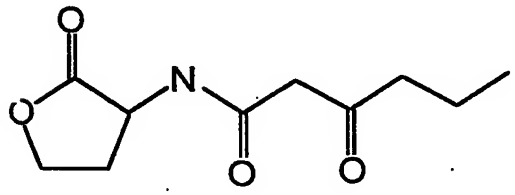
Figure 1.

a)



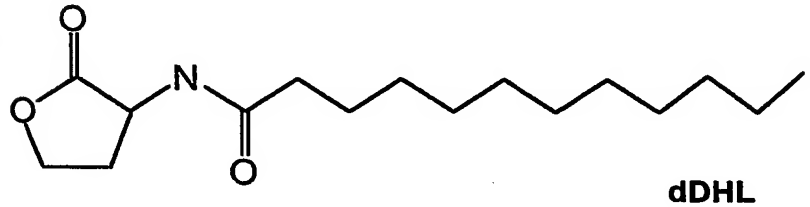
tDHL

b)



OHHL

c)



dDHL

Table 1.

scAb	dDHL	tDHL	OHHL	dDHL-BSA	Paraquat
G3G2	11 μM	21 μM	17 mM	0.28 μM	N/D
G3B12	4 μM	5 μM	2 mM	0.32 μM	N/D

N/D indicates that no IC_{50} value could be determined.

Table 2.

scAb	ka (mol l^{-1} s^{-1})	kd (s^{-1})	KA (mol/l)	KD (nM)
G3G2	4.19×10^{4}	1.43×10^{-3}	2.93×10^{7}	34.1
G3B12	3.93×10^{4}	1.56×10^{-3}	2.52×10^{7}	39.7

Figure 2.

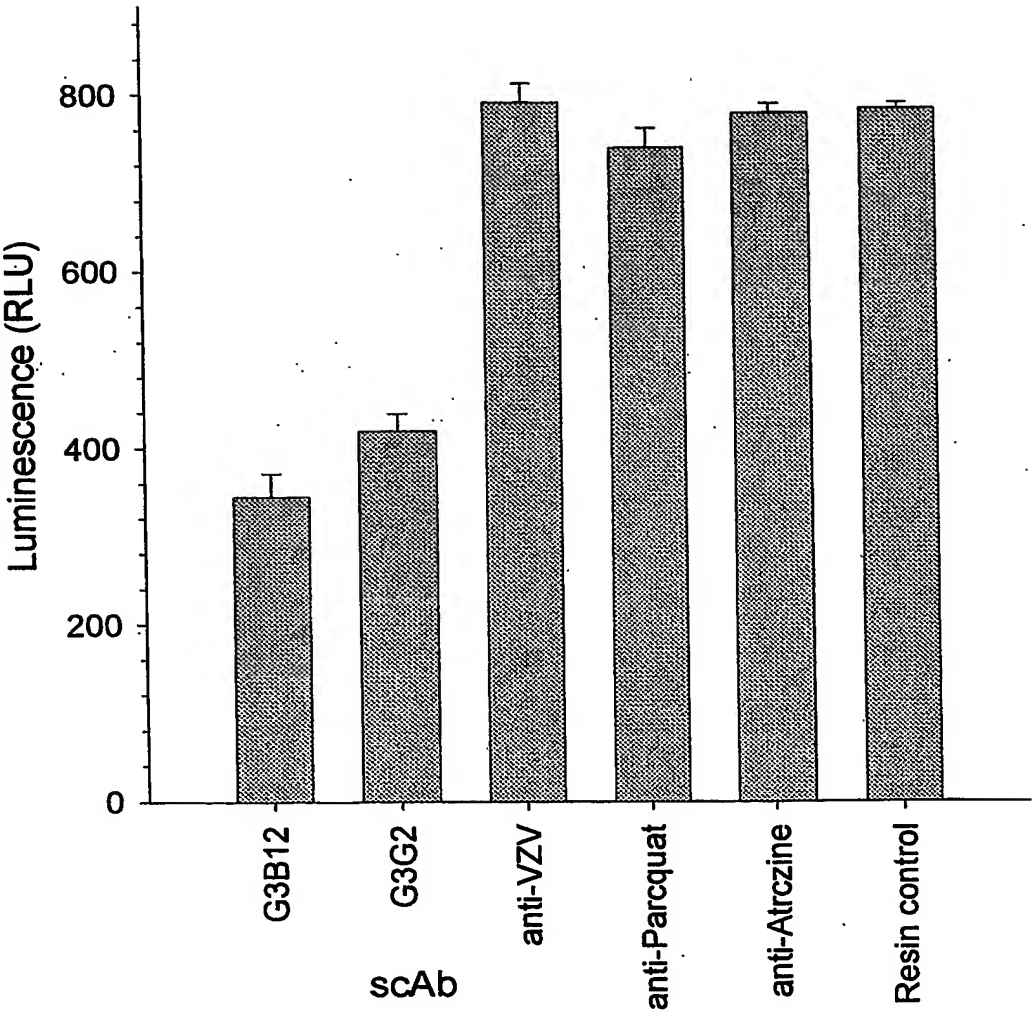

Luminescence (RLU)
800
600
400
200
0
G3B12
G3G2
anti-VZV
anti-Parcquat
anti-Atrczine
Resin control
scAb

Figure 3.

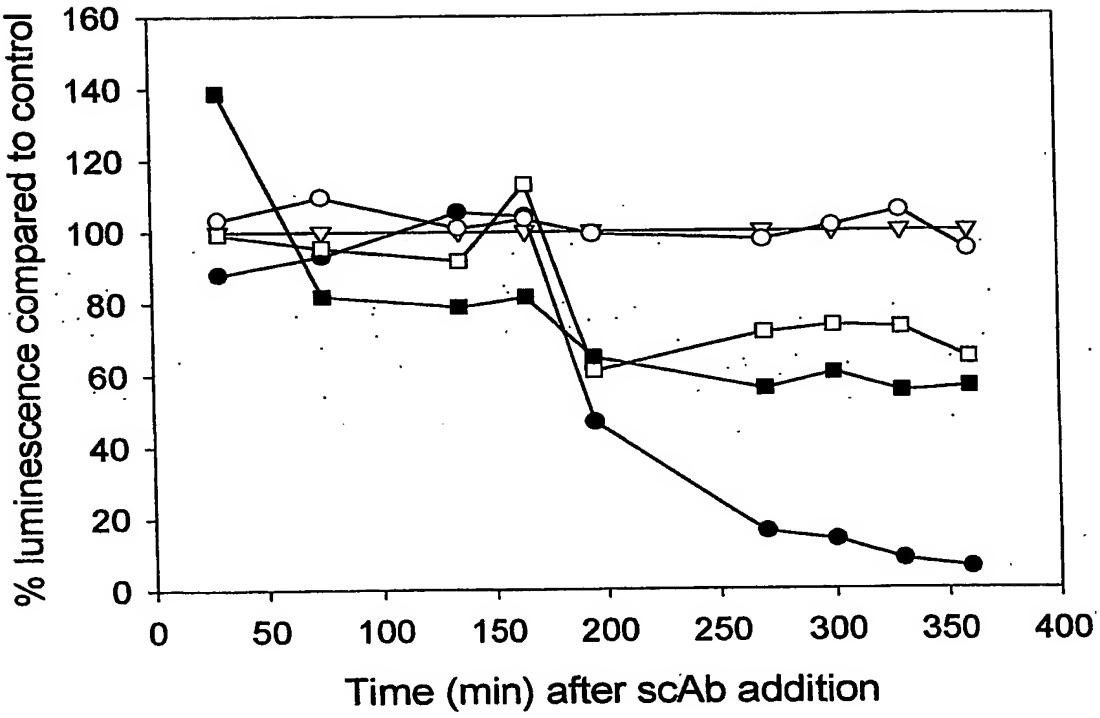

% luminescence compared to control
160
140
120
100
80
60
40
20
0
0
50
100
150
200
250
300
350
400
Time (min) after scAb addition

Figure 4.

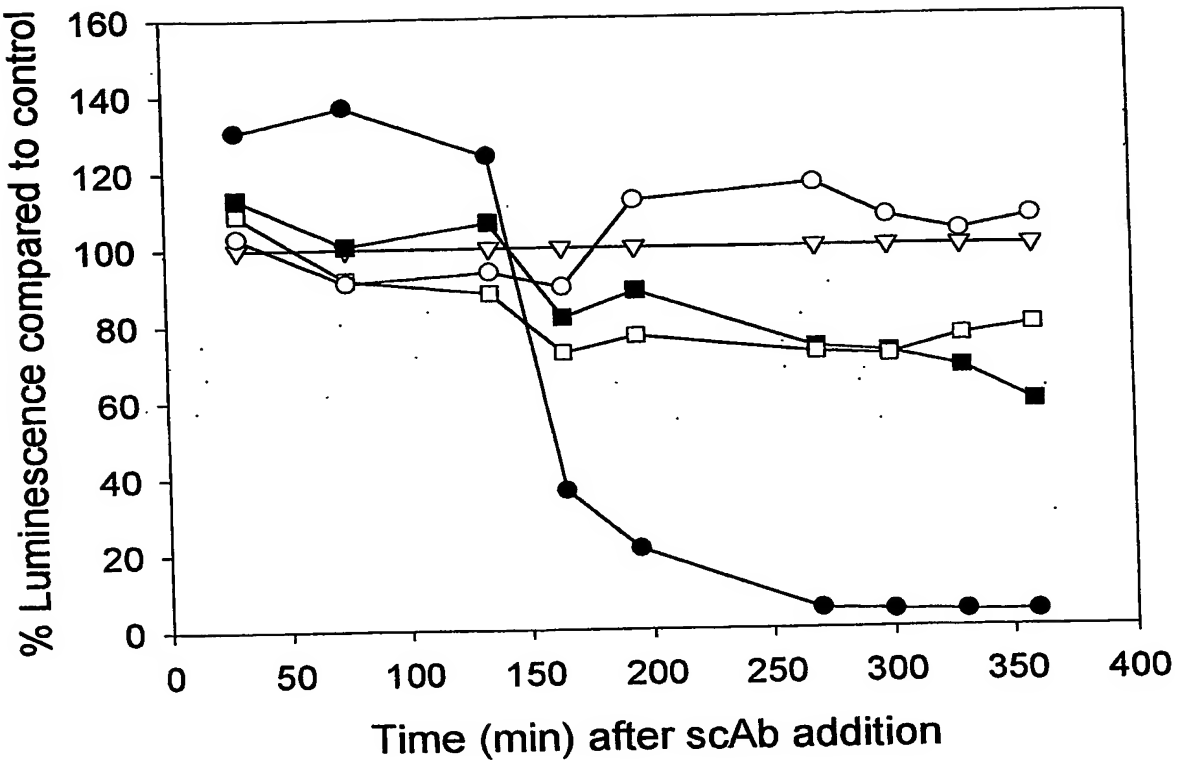

% Luminescence compared to control
160
140
120
100
80
60
40
20
0
0
50
100
150
200
250
300
350
400
Time (min) after scAb addition

Figure 1.

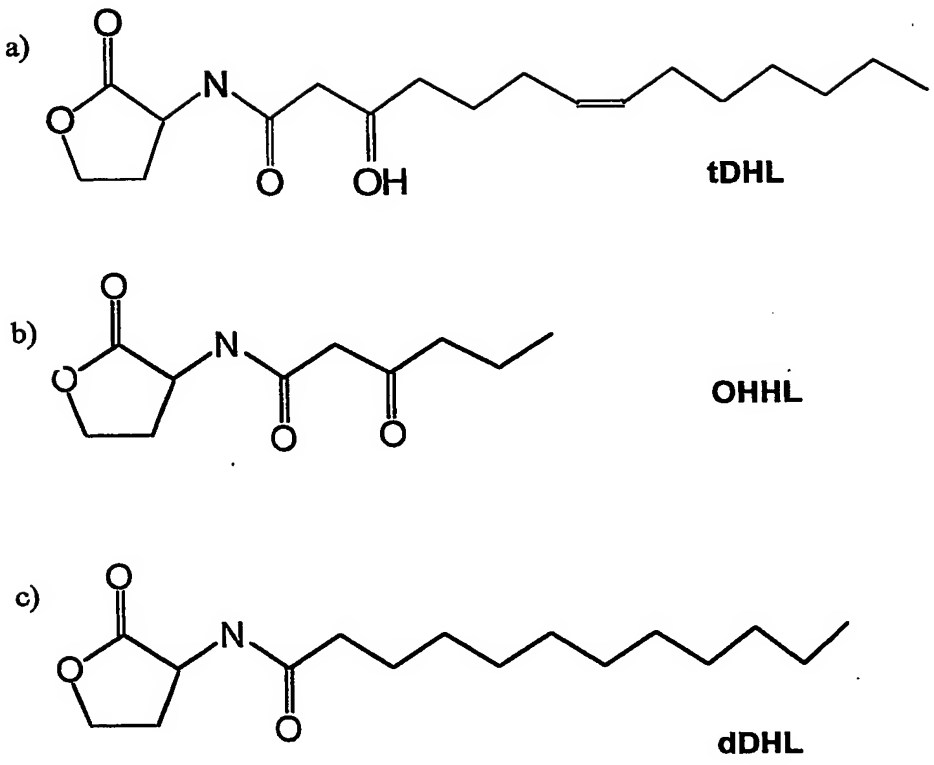


Table 1.

scAb	dDHL	tDHL	OHHL	dDHL-BSA	Paraquat
G3G2	11 μM	21 μM	17 mM	0.28 μM	N/D
G3B12	4 μM	5 μM	2 mM	0.32 μM	N/D

N/D indicates that no IC_{50} value could be determined.

Table 2.

scAb	ka (mol l^{-1} s^{-1})	kd (s^{-1})	KA (mol/l)	KD (nM)
G3G2	4.19×10^{4}	1.43×10^{-3}	2.93×10^{7}	34.1
G3B12	3.93×10^{4}	1.56×10^{-3}	2.52×10^{7}	39.7

Figure 2.

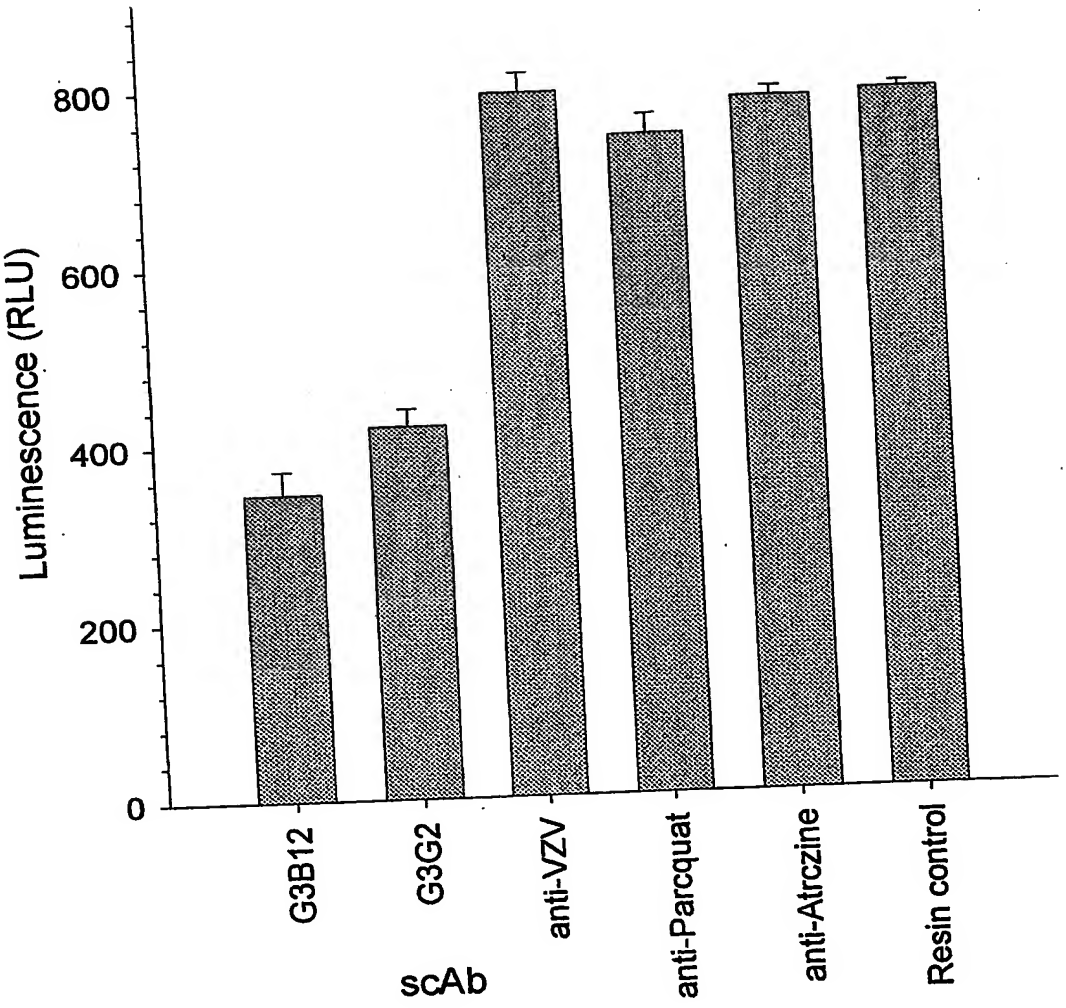

Luminescence (RLU)
0
200
400
600
800
G3B12
G3G2
anti-VZV
anti-Parcquat
anti-Atrczine
Resin control
scAb

Figure 3.

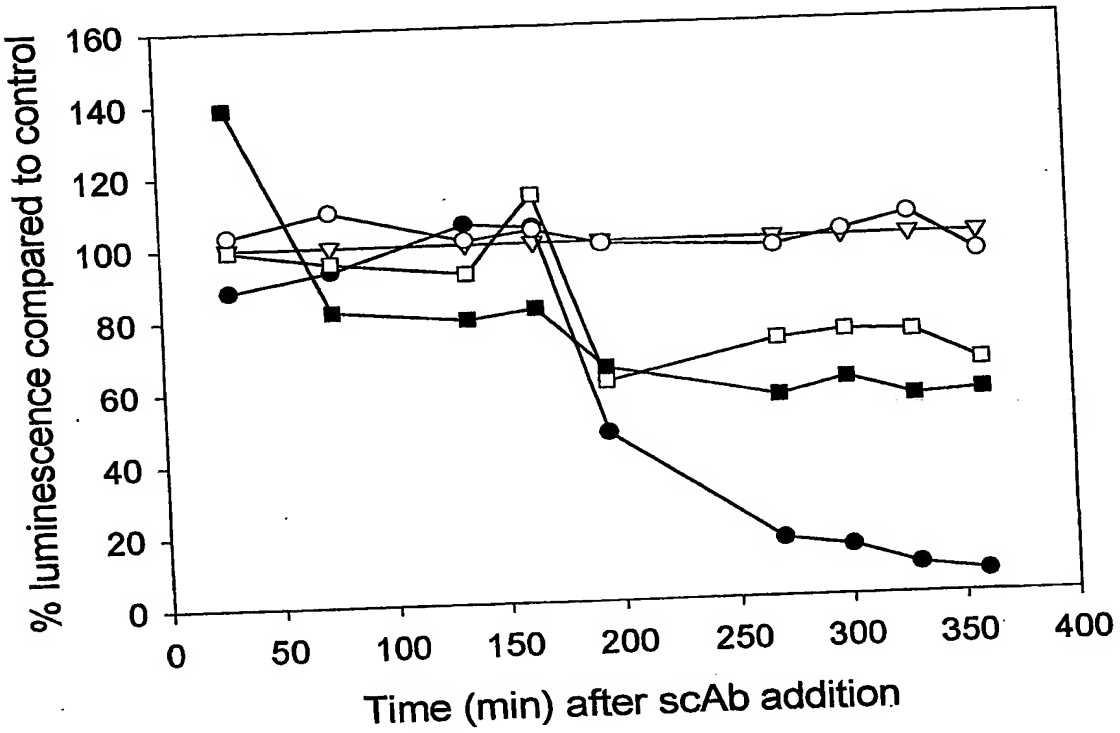
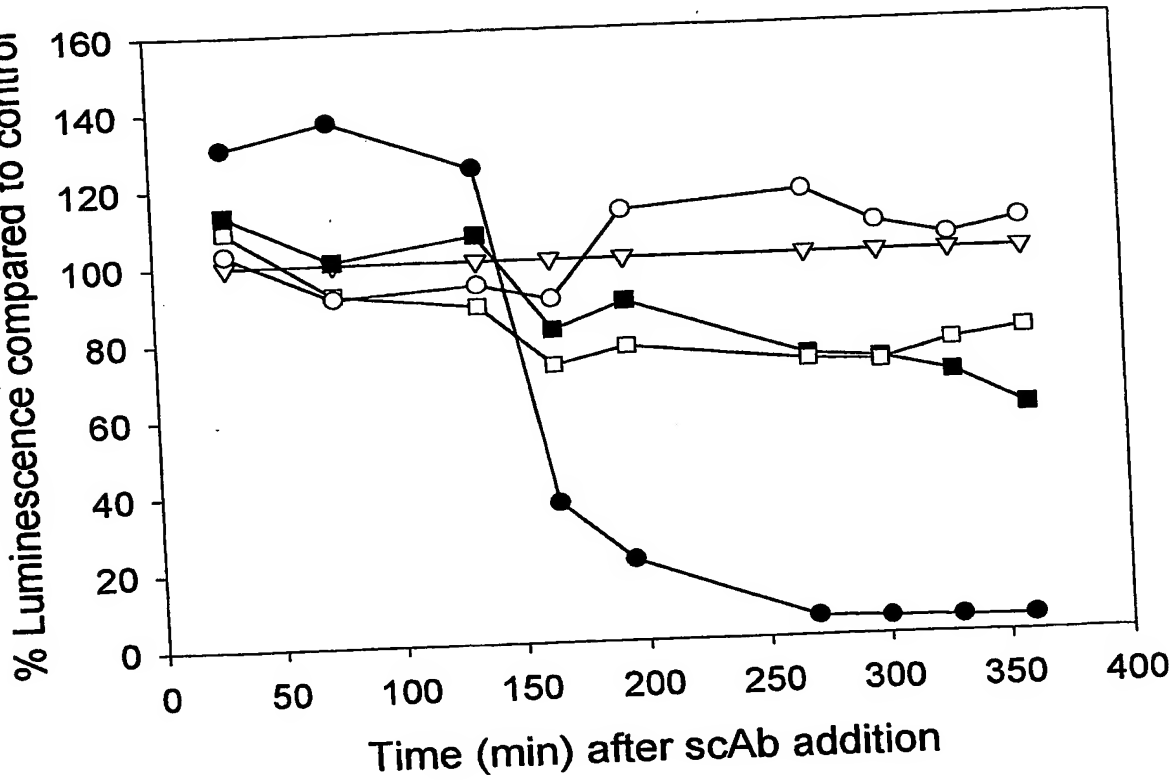

% luminescence compared to control
160
140
120
100
80
60
40
20
0
0
50
100
150
200
250
300
350
400
Time (min) after scAb addition

Figure 4.


% Luminescence compared to control
160
140
120
100
80
60
40
20
0
0
50
100
150
200
250
300
350
400
Time (min) after scAb addition

References cited

U.S. Patent Documents

6,309,651	October 2001	Frank et al.

Other Patent Documents

WO 01/26650	April 2001	University of Nottingham
WO 01/74801	October 2001	University of Nottingham
WO 92/01047	October 2001	Bonnert et al.

Other References

Williams et al., 1996 Microbiol-UK 142: 881-888
Stintzi et al., 1998 FEMS Microbiol Lett. 166 (2): 341-345
Glessner et al., 1999 J. Bacteriol. 181 (5): 1623-1629
Brint and, Ohman 1995 J. Bacteriol. 177 (24): 7155-7163
Reimmann et al., 1997 Mol. Microbiol. 24 (2): 309-319
Winzer et al., 2000 J. Bacteriol. 182 (22): 6401-6411
Gambello and Iglewski 1991 J. Bacteriol. 173 (9): 3000-3009
Latifi et al., 1995 Mol. Microbiol 17 (2): 333-343
Passador et al., 1993 Science 260: 1127-1130
Pearson et al., 1994 Proc. Natl. Acad. Sci. USA. 91 (1): 197-201
Winson et al., 1995 Proc. Natl. Acad. Sci. USA. 92 (20): 9427-9431
Pesci et al., 1997 J. Bacteriol. 179 (10): 3127-3132
Toder et al., 1991 Mol. Microbiol. 5 (8): 2003-2010
Gambello et al., 1993 Infect. Immun. 61 (4): 1180-1184
Pearson et al., 1995 Proc. Natl. Acad. Sci. USA 92 (5) 1490-1494
Latifi et al., 1996 Mol. Microbiol 21 (6): 1137-1146
Winzer et al., 2000 J. Bacteriol. 182 (22): 6401-6411
Manefield et al., 1999 Microbiol. UK 145: 283-291
Tepletski et al., 2000 Mol. Plant Microbe. Interact., 13, 637-648
McCafferty et al., 1990 Nature 348: 552-554
Huston et al., 1993 Int. Rev. Immunol., 10, 195-217
Hart et al., 2000 J. Am. Chem. Soc., 122, 460-465
Whitcombe et al., 1995 J. Am. Chem. Soc., 117, 7105-7111
Ramstrom et al., 1996 Chem. & Biol., 3, 471-477
Rachkov et al., 2000 Anal. Chim. Acta. 405, 23-29